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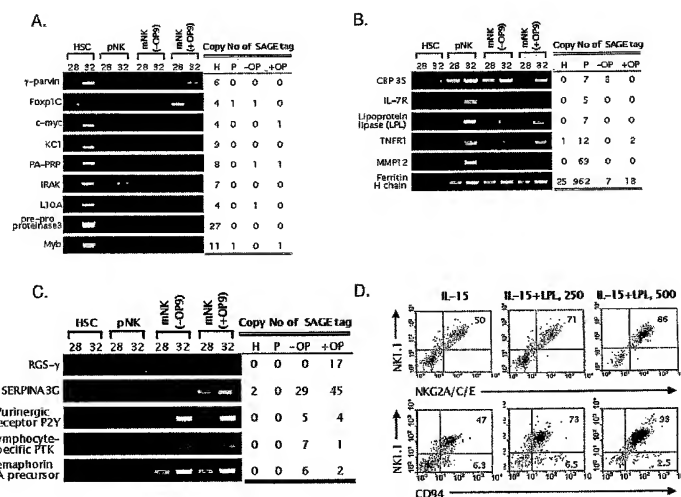
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(54) Title: DIFFERENTIATION REGULATING AGENT CONTAINING GENE WHICH REGULATING DIFFERENTIATION FROM STEM CELLS INTO NATURAL KILLER CELLS AS EFFECTIVE INGREDIENT



(57) Abstract: The present invention relates to a cell differentiation regulating agent containing a gene regulating differentiation from stem cells into natural killer cells as an effective ingredient, more precisely, a cell differentiation regulating agent containing a gene regulating differentiation from stem cells into premature natural killer cells as an effective ingredient and a screening method of the gene by taking advantage of SAGE. The gene of the present invention is a novel one that is confirmed not to be like any other known genes regulating differentiation from stem cells into natural killer cells. Though, the gene can be easily screened by SAGE and a natural killer cell differentiation-regulating agent containing the gene as an effective ingredient can be effectively used as an anticancer agent.

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【DESCRIPTION】

【Invention Title】

DIFFERENTIATION REGULATING AGENT CONTAINING GENE
WHICH REGULATING DIFFERENTIATION FROM STEM CELLS INTO
5 NATURAL KILLER CELLS AS EFFECTIVE INGREDIENT

【Technical Field】

The present invention relates to a differentiation-
regulating agent containing a gene regulating
10 differentiation from stem cells into natural killer cells
as an effective ingredient and a screening method for the
gene.

【Background Art】

15 Stem cells have multipotency for the differentiation
into various organs and have self-renewal capacity, and
are found in both embryos and adults. The stem cells
enable differentiation of a cell into a specific cell or
an organ, so that our attention has been focused on the
20 possibility to use the stem cells for organ
transplantation or cell therapy.

Hematopoietic stem cells, a kind of adult stem cells,
are the cells that can be differentiated into every blood

forming cells such as erythrocytes, leucocytes, platelets and lymphocytes. And cells involved in immune system are continuously self-renewed from the hematopoietic stem cells in bone marrow. Hematopoietic stem cells have been
5 used so far for the treatment of various blood diseases including cancer by means of bone marrow transplantation. According to recent reports, the hematopoietic stem cells could be differentiated into other types of cells such as muscle, nerve, bone, etc, in animal models. If they can
10 be applied to human, the hematopoietic stem cells can be used for the treatment of in variety of diseases including diabetes, Parkinson's disease, spinal cord injury, etc, because they can replace other cells and organs successfully.

15 In particular, natural killer (referred as 'NK' hereinafter) cells destroy cancer cells non-specifically. Owing to their cytotoxic capacity, NK cells have now been in use for the treatment of a solid tumor using LAK (lymphokine activated killer cell) and TIL (tumor
20 infiltration lymphocytes) and for immune therapy (J Immunol., 1986, 36(10):3910-3915; Hematologia, 1999, 84:1110-1149) using donor lymphocyte infusion, suggesting that it further makes the way to new cell therapy to reduce rejection after bone marrow transplantation or
25 organ transplantation. It was also reported that the

defect in differentiation and activation of NK cells is related to various diseases including breast cancer (Breast Cancer Res Treat., 2003, 66(3):255-263), melanoma (Melanoma Res., 2003, 13(4):349-356) and lung cancer (Lung Cancer, 2002, 35(1):23-18), so that NK cell therapy draws our attention to treat such diseases.

Thus, the present inventors have identified a novel gene regulating differentiation of stem cells into NK cells by using SAGE (Serial Analysis of Gene Expression) and have completed this invention by confirming that NK cell differentiation is regulated by the gene above and further the gene can be a great aid for the treatment of diseases including cancer.

【Disclosure】

【Technical Problem】

It is an object of the present invention to provide a NK cell differentiation-regulating agent containing a gene which regulating differentiation from stem cells into natural killer cells as an effective ingredient and a screening method for the gene using SAGE.

【Technical Solution】

In order to achieve the above object, the present invention provides a differentiation-regulating agent which regulates differentiation from stem cells into natural killer cells.

The present invention also provides a differentiation-regulating agent which regulates differentiation from stem cells into premature natural killer cells.

The present invention further provides a differentiation-regulating agent which regulates differentiation from premature natural killer cells into mature natural killer cells.

The present invention also provides an anticancer agent developed by using the differentiation-regulating agent of the invention.

The present invention further provides a screening method for a gene regulating differentiation from stem cells into natural killer cells, based on SAGE.

In the present invention, 'differentiation regulating gene' means every gene that regulate differentiation from stem cells into natural killer cells, that is, they can accelerate or inhibit differentiation.

More precisely, differentiation-regulating gene of the present invention can accelerate differentiation, so that it promotes a progress to the next stage. In the meantime, it also has functions of maintaining each stage or inhibiting a progress to the next stage.

In the present invention, 'SAGE' stands for 'serial analysis of gene expression'. SAGE can be performed either by conventional method or by manufacturer's protocol (InvitrogenTM life technologies) (<http://www.invitrogen.com>).

The mark in bracket after the name of gene means GenBank ID implying sequence of each gene and the GenBank ID can be easily searched and used by the people in this field.

Type II restriction enzyme used in the present invention is a conventional enzyme widely used in the field of genetic engineering. It needs magnesium ions to activate and recognizes a specific nucleotide sequence of DNA, so that it can cut exactly the wanting area or the neighboring area apart from the recognized nucleotide sequence. Type II S restriction enzyme used in the present invention means NlaIII (recognizes and digests the area of CATG region every 250 base pairs).

Hereinafter, the present invention is described in

detail.

The present invention provides a differentiation regulating agent for natural killer cells which is characterized by containing one or more genes, as an effective ingredient, selected from a group consisting of homeobox protein MIX (AF15457), pre-pro-proteinase 3 (U97073), myeloblastosis (Myb) oncogene (M16499), keratin complex 1, acidic, gene 13 (NM_010662), PA-phosphatase related phosphoesterase (AK002966), gamma-parvin (BC011200), forkhead-related transcription factor 1C (AF330105), RIKEN cDNA 5730501N20 gene (AK017744), c-myc protein (X010223), ribosomal protein L10A (AK002613), Oct 2b gene (X53654), microlite (AK015601), dihydrolipoamide dihydrogenase (BC003368), tracle (U81030), lysozyme (BC002069), ferritin H chain (BC012314), brevican (X87096), matrix metalloproteinase 12 (BC019135), EIA-stimulated gene cellular inhibitor (AF084524), S100 calcium binding protein A9 (BC027635), MPS1 protein (L20315), transglutaminase 2 (BC016492), serum and glucocorticoid regulated protein kinase (AF139639), RIKEN cDNA 5830413L19 (BC027496), interferon-induced protein (BC003804), milk fat globule membrane protein EGF factor 8 (BC018577), cell-surface glycoprotein p91 (U83172), arginase 1 (BC050005), tumor necrosis factor receptor 1 (M59378), retinoid-induced serine carboxypeptidase (AF330052),

FLJ11000 homologue (BC023802), interleukin-18 binding
protein d precursor (AF110803), chloride channel 7
(AK009435), CD36 antigen (BC010262), zink finger protein
homologue (BC030186), carbohydrate binding protein 35
5 (J03723), C-type calcium dependent carbohydrate (BC003218),
lipoprotein lipase (NM_008509), v-maf lacertus
fibrosarcoma oncogene (BC038256), interleukin 7 receptor
(NM_008372), chemokine (C-C) receptor 1 (BC011092),
neurophilin (MGD|MGI:106206) (AK002673), SERPINA3G
10 (XM_127137), GABA-A receptor subunit 6 (X51986), LAPTm5
(U51239), G-protein signal regulator (BC049968), decoy-
stimulating factor GPI fixed mRNA (L41366), Y box protein
3 (AK019465), osteopontin precursor (J04806), amyloid beta
(A4) precursor protein-binding family (AK021331), T cell
15 receptor beta subunit analogue (U63547), immune related
nucleotide 1 (BC005577), higher stage transcription factor
1 (NM_009480), olfactory receptor MOR267-7 (NM_146714),
lymphocyte specific protein tyrosine kinase (M12056),
osteoclast cancer inhibitor (AB013898), platelet active
20 receptor homologue (BC024054), natural killer cell protein
2-A1 (AF016008), unidentified protein MGC36662 (BC023851),
semaphorin 6A precursor homologue (AK004390),
neurofilament homologue polypeptide (BC025872), cornin
homologue actin binding protein 2A (BC026634), solute
25 transmitting family 6 (BC015245), temporary purine

receptor P2Y10 homologue (AK020001), T cell receptor gamma chain (X03802), poly A polymerase alpha (NM_011112), OPA-related protein OIP5 analogue (AK017825) and mytogen activated protein kinase 1 analogue (BC006708).

5 The present invention also provides a differentiation regulating agent which regulates differentiation from stem cells into premature natural killer cells which is characterized by containing one or more genes selected from a group consisting of homeobox protein MIX (AF15457), pre-pro-proteinase 3 (U97073),
10 myeloblastosis (Myb) oncogene (M16499), keratin complex 1, acidic, gene 13 (NM_010662), PA-phosphatase related phosphoesterase (AK002966), gamma-parvin (BC011200), forkhead-related transcription factor 1C (AF330105), RIKEN
15 cDNA 5730501N20 gene (AK017744), c-myc protein (X010223), ribosomal protein L10A (AK002613), Oct 2b gene (X53654), microlite (AK015601), dihydrolipoamide dihydrogenase (BC003368) and tracle (U81030), as an effective ingredient.

 The present invention further provides a
20 differentiation regulating agent which regulates differentiation from premature natural killer cells into mature natural killer cells which is characterized by containing one or more genes, as an effective ingredient, selected from a group consisting of lysozyme (BC002069),
25 ferritin H chain (BC012314), brevican (X87096), matrix

metalloproteinase 12 (BC019135), EIA-stimulated gene
cellular inhibitor (AF084524), S100 calcium binding
protein A9 (BC027635), MPS1 protein (L20315),
transglutaminase 2 (BC016492), serum and glucocorticoid
5 regulated protein kinase (AF139639), RIKEN cDNA 5830413L19
(BC027496), interferon-induced protein (BC003804), milk
fat globule membrane protein EGF factor 8 (BC018577),
cell-surface glycoprotein p91 (U83172), arginase 1
(BC050005), tumor necrosis factor receptor 1 (M59378),
10 retinoid-induced serine carboxypeptidase (AF330052),
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15 (J03723), C-type calcium dependent carbohydrate (BC003218),
lipoprotein lipase (NM_008509), v-maf lacertus
fibrosarcoma oncogene (BC038256), interleukin 7 receptor
(NM_008372), chemokine (C-C) receptor 1 (BC011092) and
neurophilin (MGD|MGI:106206).

20 The present invention also provides a
differentiation regulating agent which regulates
differentiation of mature natural killer cells which is
characterized by containing one or more genes, as an
effective ingredient, selected from a group consisting of
25 SERPINA3G (XM_127137), GABA-A receptor subunit 6 (X51986),

LAPTM5 (U51239), G-protein signal regulator (BC049968),
decoy-stimulating factor GPI fixed mRNA (L41366), Y box
protein 3 (AK019465), osteopontin precursor (J04806),
amyloid beta (A4) precursor protein-binding family
5 (AK021331), T cell receptor beta subunit analogue (U63547),
immune related nucleotide 1 (BC005577), higher stage
transcription factor 1 (NM_009480), olfactory receptor
MOR267-7 (NM_146714), lymphocyte specific protein tyrosine
kinase (M12056), osteoclast cancer inhibitor (AB013898),
10 platelet active receptor homologue (BC024054), natural
killer cell protein 2-A1 (AF016008), unidentified protein
MGC36662 (BC023851), semaphorin 6A precursor homologue
(AK004390), neurofilament homologue polypeptide (BC025872),
cornin homologue actin binding protein 2A (BC026634),
15 solute transmitting family 6 (BC015245), temporary purine
receptor P2Y10 homologue (AK020001), T cell receptor gamma
chain (X03802), poly A polymerase alpha (NM_011112), OPA-
related protein OIP5 analogue (AK017825) and mytogen
activated protein kinase 1 analogue (BC006708).

20

A gene included in the differentiation regulating
agent of the present invention has functions of 1)
regulating differentiation from stem cells into premature
NK cells, 2) regulating differentiation from premature NK
25 cells into mature NK cells, and 3) regulating

differentiation of mature NK cells, and a differentiation regulating gene functioning at each stage can be independently used as a differentiation regulating agent from stem cells into NK cells. In the preferred embodiment of the present invention, differentiations from stem cells into premature NK cells and into mature NK cells were induced by culturing HSC cells with the treatment of cytokine (FIG. 1a - FIG. 1c). From each stage, a whole RNA was separated and SAGE was performed as shown in a schematic diagram of FIG. 2. By SAGE, genes showing a specific increase of expression were selected from each differentiation stages (FIG. 3a - FIG. 3f). The genes were compared with others deposited at GenBank. As a result, the genes were none of those reported to have functions of regulating differentiations from stem cells into pNK cells (see Table 3), from pNK cells into mNK cells (see Table 4) and of mNK cells (see Table 5).

Therefore, the genes of the present invention are a new founding having a novel differentiation regulating mechanism, and a pharmaceutical composition having one or more of those genes can be used for the regulation of cell differentiation. In particular, a differentiation regulating agent involved in the differentiation from stem cells into premature NK cells can be prepared by using one or more of genes listed in Table 3, and also a

differentiation regulating agent involved in the differentiation from premature NK cells into mature NK cells can be prepared by using one or more genes listed in Table 4. A differentiation-regulating agent involved in the differentiation of mature NK cells can be prepared by using one or more genes listed in Table 5. All the genes listed in Table 3, 4 and 5 have functions of regulating the differentiation from stem cells into NK cells, so a differentiation regulating agent which regulates differentiation of natural killer cells can be prepared by using one or more genes mentioned above.

Cell differentiation regulating agent of the present invention can also be used for the treatment of cancers. The differentiation-regulating agent of the invention is preferably applicable to such cancers as breast cancer, melanoma and lung cancer. The defects of NK cell differentiation and activation result in various cancers, for example, breast cancer (Breast Cancer Res Treat., 2003, 66(3):255-263), melanoma (Melanoma Res., 2003, 13(4):349-356) and lung cancer (Lung Cancer, 2002, 35(1):23-18). Thus, the mentioned cancers can be effectively treated by regulating NK cell differentiation with the NK cell differentiation-regulating agent of the present invention.

The cell differentiation-regulating agent of the

present invention can be administered orally or parenterally and be used in general forms of pharmaceutical formulation. The cell differentiation-regulating agent of the present invention can be prepared
5 for oral or parenteral administration by mixing with generally used fillers, extenders, binders, wetting agents, disintegrating agents, diluents such as surfactant, or excipients. Solid formulations for oral administration are tablets, pill, dusting powders, granules and capsules.
10 These solid formulations are prepared by mixing with one or more suitable excipients such as starch, calcium carbonate, sucrose or lactose, gelatin, etc. Except for the simple excipients, lubricants, for example magnesium stearate, talc, etc, can be used. Liquid formulations for
15 oral administrations are suspensions, solutions, emulsions and syrups, and the abovementioned formulations can contain various excipients such as wetting agents, sweeteners, aromatics and preservatives in addition to generally used simple diluents such as water and liquid
20 paraffin. Formulations for parenteral administration are sterilized aqueous solutions, water-insoluble excipients, suspensions, emulsions, and suppositories. Water insoluble excipients and suspensions can contain, in addition to the active compound or compounds, propylene
25 glycol, polyethylene glycol, vegetable oil like olive oil,

injectable ester like ethylolate, etc. Suppositories can contain, in addition to the active compound or compounds, witepsol, macrogol, tween 61, cacao butter, laurin butter, glycerol, gelatin, etc.

5 The effective dosage of the agent of the present invention is 0.1 ~ 0.2 mg/kg, and preferably 0.15 mg/kg. The administration times of the agent of the present invention might be once to three times a day.

10 The present invention also provides a screening method for a gene regulating the differentiation from stem cells into natural killer cells comprising the following steps:

15 1) Synthesizing cDNA after separating whole RNA from cells;

2) Separating tag after digesting the cDNA of the step 1;

3) Connecting each tag separated in the step 2 and then analyzing nucleotide sequence thereof; and

20 4) Quantifying the expression of the gene, based on the analyzed nucleotide sequence above, by using SAGE analyzing program.

25 In the step 1, cells are preferably selected from each stage of differentiation from stem cells into natural

killer cells. In the preferred embodiment of the present invention, hematopoietic stem cells (HSC) were used as stem cells, and premature natural killer cells and mature natural killer cells were used as natural killer cells.

5 Any conventional method, if only it is able to separate whole RNA from sample with high yield and with preventing RNase contamination, can be used (Sambrook, et al., 1989, Molecular Cloning). In general, it is easy to follow manufacturer's protocol to separate RNA by using a RNA
10 separating agent. In order to synthesize cDNA from a whole RNA, oligo dT primer was attached to a whole RNA, but that was not the only way to synthesize cDNA and any other method to synthesize cDNA could be used. In the preferred embodiment of the present invention, oligo dT
15 primer was attached to a whole RNA to synthesize cDNA and at that time, oligo dT primer was to insert poly A sequence for the synthesis of mRNA. It is preferred that 20 - 30 T sequences are repeated in oligo dT primer. And, it is also allowed that magnetic beads are additionally
20 attached to one end of the oligo dT primer, because tag can be successfully separated without contamination by using magnetic beads.

In the step 2, the process of separating tag after
25 digesting the cDNA is composed of the following steps:

a) Preparing tag by digesting cDNA with II S type restriction enzyme 1;

b) Combining two kinds of adapters each including a II S type restriction enzyme 1 recognition site at one end
5 cleavage site of the tag prepared in the step a;

c) Separating tag by digesting the tag connected to the adapter in the step b with II S type restriction enzyme 2 and cutting off oligo dT magnetic beads from the tag;

d) Preparing ditag by combining the tags prepared in
10 the step c each other; and

e) Preparing ditag only by digesting the ditag prepared in the step d with II S type restriction enzyme 1 and cutting off the adapter.

15 In the step a, the reason why the synthesized cDNA was digested with II S type restriction enzyme 1 was that the cleavage site digested by the enzyme could be prepared as a tag binding site and in fact it was easy to use the area for binding with tag because the cleavage site formed
20 5' overhangs. As a II S type restriction enzyme 1, any adequate enzyme is possible and NlaIII restriction enzyme is preferred. That is because cDNA has NlaIII restriction enzyme recognition sites at every 250 bp, so that regular sized tag can be easily prepared by digesting cDNA with
25 the enzyme.

In the step b, two kinds of adapters to be linked to the cleavage site of tag have about 40 bp long sequences that are bound each other complementarily. The adapters include NlaIII restriction enzyme recognition site (CATG) at one end, to which tag is bound, and form overhangs which make the bond with tag easy.

In the step c, the tag bound to the adapter was digested with II S type restriction enzyme 2. II S type restriction enzyme 2 was bound to the restriction enzyme site of an adapter to cut the area located at 10 - 14 bp downstream from the restriction enzyme cleavage site, resulting in the separation of about 50 bp long tag containing the end of 4 bp size overhang at 5' end. BsmFI was preferably used as a II S type restriction enzyme 2.

In the step d, tags were connected each other to form a ditag. Precisely, the end of overhang was formed at each 5' end of the tags, so that a ditag could be easily formed by connecting those ends. The resultant ditag was about 100 pb long.

In the step e, the ditag was digested with II S type restriction enzyme 1 to cut the adapter off, resulting in pure ditag only. Precisely, the binding area where the end of tag and an adapter were bound included II S type restriction enzyme recognition site, so the adapter could be cut off by using the II S type restriction enzyme 1. As

a result, about 26 bp long pure ditag was prepared.

In the mean time, in the step 3, 10 to 20 tag fragments, obtained in the step 2, were bound and their nucleotide sequences were investigated. And the investigation process was composed of the following steps:

a) Cloning the concatemer type ditag prepared by binding ditags prepared in step 2 into a vector; and

b) Investigating nucleotide sequence of tag of the vector used for cloning in step a.

In the step a, ditags were bound to form a concatemer. Precisely, both ends of a ditag included II_S type restriction enzyme 1 recognition site, indicating that overhang could be formed. Such ditags could be connected easily and so about 20 to 50 tags were connected to form a concatemer. The prepared concatemer type tag was inserted into a conventional vector for cloning to investigate nucleotide sequence thereof. In the preferred embodiment of the present invention, pZerO-1 vector was used for the cloning. The mentioned expression vector was included in a kit (Invitrogen Life Science) provided for SAGE analysis and was very useful.

In the step 4, the expression was quantified by investigating nucleotide sequence obtained above with SAGE

analyzing program. Precisely, the obtained nucleotide sequence was compared with other sequences of genes deposited at GenBank to identify it. Then, SAGE analyzing program was used to classify sequences from ones with high expression to others with low expression. They were marked with red, yellow, green and blue after clustering, making the expression levels be shown clearly. And the amount of expression can be evaluated as a numerical value. SAGE analyzing program can be either provided by a company or one of soft wares provided through internet. In the present invention, a conventional program (cluster and treeview computer program, <http://rana.lbl.gov>) widely used for clustering of SAGE results was used.

A screening method of the present invention is based on SAGE analysis. Each step of the method was performed by taking advantage of the general SAGE analysis or could be performed by modified processes according to manufacturer' instruction. The outline of the method of the invention is shown in a schematic diagram of FIG. 2.

【Description of Drawings】

FIG. 1a - FIG. 1c show the comparison of expressions of surface molecules during the differentiation processes from mouse hematopoietic stem cells (HSC) through

premature NK cells (pNK) to mature NK cells (mNK) in the presence (+OP9) or in the absence (-OP9) of OP9 interstitial cells.

FIG. 1a is a set of graphs showing the purity of cells of each stage of NK cell differentiation which was presented by two different colors determined by flow cytometry. The numbers of each quadrant indicate percentage of corresponding cells.

Lin- c-kit+: (96%), CD122+ NK1.1-: (95%),
CD122+ NK1.1+: (94%, 95% respectively)

FIG. 1b is a set of graphs showing the expressions of NK cell related surface markers (NK1.1, DX5, CD94, NKG2A) induced during the differentiation from premature NK cells into mature NK cells, for which OP9 interstitial cells were added for the culture.

FIG. 1c is a set of photographs showing the results of RT-PCR. Whole cytoplasmic RNA was extracted from cells from each stage of NK cell differentiation to investigate whether or not CD122, a representative NK cell related gene, and perforin were expressed.

FIG. 2 is a schematic diagram showing the SAGE process to detect a differentiation-regulating gene of the present invention.

FIG. 3a - FIG. 3f show clustering of gene expression profile obtained during NK cell differentiation by using

SAGE analysis.

FIG. 3a shows the group of genes expressed most in HSC cells, FIG. 3b presents the group of genes expressed most in pNK cells, FIG. 3c shows the group of genes expressed most in mNK (-OP9) cells, and FIG. 3d presents the group of genes expressed most in mNK (+OP9) cells.

FIG. 3e shows genes inhibiting the activation of NK cells, and FIG. 3f shows genes promoting the activation of NK cells.

In the above FIG. 3a - FIG. 3f, from the clustering based on SAGE analysis, when the cluster frequency was over 80, it was marked red, when the frequency was 50 - 79, it was marked yellow, when the frequency was 30 - 49, it was marked green and when the frequency was under 29, it was marked blue.

FIG. 4a - FIG. 4d show the results of RT-PCR to investigate whether the gene that was confirmed by SAGE to regulate the differentiation of NK cells was actually expressed. The expression was quantified in comparison with the comparative beta-actin gene.

FIG. 4a shows genes expressed specifically in HSC cells during the NK cell differentiation, FIG. 4b presents genes expressed specifically in pNK cells, FIG. 4c shows genes expressed specifically in mNK cells, and FIG. 4d shows that LPL was treated to NK cells at different

concentrations (250 ng/ml and 500 ng/ml) to investigate the effect of LPL on the differentiation of NK cells, and as a result, the differentiation into mNK cells was promoted.

5 **【Mode for Invention】**

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

10 However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

<Example 1> Stem cell isolation from bone marrow

15 All bones including tibia and femur of a C57BL/6 mouse (Dae Han Biolink) at the age of 6 - 9 weeks were pulverized. The pulverized pieces were passed through 70-micron cell strainer and erythrocytes in them were removed by treating lysis solution (Sigma, St. Louse, MO) to
20 obtain bone marrow cells only. The bone marrow cells were reacted with antibody markers that were biotin labeled for systemic markers (CD11b : macrophage marker, Gr-1 : granulocyte marker, B220 : B cell marker, NK1.1 : NK cell marker, CD2 : T cell marker, TER-119 : erythrocyte

marker), followed by washing. Then, the cells were reacted with streptavidin labeled magnetic beads (Miltenyi Biotec, Auburn, CA). Magnetic labeled Lin⁺ cells were scavenged by being passed through CS column (Miltenyi Biotec) in the magnetic field of MACS (Miltenyi Biotec). The remaining Lin⁻ cells passed through the column were reacted with magnetic beads connected to c-kit and then passed through MACS column (Miltenyi Biotec), resulting in c-kit⁺ cells remaining in the column. The purity of the obtained Lin⁻ c-kit⁺ hematopoietic stem cells.(referred as 'HSC cells' hereinafter) was measured by FACS (BD Bioscience, Mountainview, CA). As a result, it was confirmed that the cells had over 96% purity.

<Example 2> Inducement of the differentiation from stem cells into NK cells

HSC cells, separated from bone marrow in the Example 1, in RPMI complete medium supplemented with mouse SCF (30 ng/ml, BioSource, Camarillo, CA), mouse Flt3L (50 ng/ml, PeproTech, Rocky Hill, NJ), mouse IL-7 (0.5 ng/ml, PeproTech), indomethacin (2 µg/ml, Sigma), gentamycin (20 µg/ml) and 10% fetal bovine serum were inoculated to a 6-well plate (Falcon) at the concentration of 2×10^6 cells/well. The cells were cultured in a 37°C, 5% CO₂

incubator for 6 days. After 3 days from the culture, half of the supernatant was discarded and a fresh medium supplemented with cytokine along with the same composition as the above was added. 6 days later, CD122+ premature NK cells (referred as 'pNK cells' hereinafter) were separated with MACS using FITC labeled CD122 antibody and magnetic beads conjugated anti-FITC antibody. The purity of the premature NK cells was measured by FACS, and from the result, it was confirmed that the cells had over 92% purity.

In order to induce the differentiation into mature NK cells (referred as 'mNK cells' hereinafter), HSC cells were recovered after 6 days from the culture, and then cultured them only or with OP9 stromal cells (Science 1994, 265(5175): 1098-1101; Nakano T, Kodama H, Honjo T.: Generation of lymphohematopoietic cells from embryonic stem cells in culture) in the presence of mouse IL-15 (20 ng/ml, PeproTech). 3 days later, half of the medium was replaced with a fresh one having the same composition. On day 12, NK1.1+ cells were separated by using FITC labeled anti-NK1.1 antibody and magnetic beads conjugated anti-FITC antibody. Mature NK cells were investigated with flow cytometry using anti-CD122, NK1.1, DX5 and NK cell receptor antibodies.

<Example 3> Investigation of purified NK cell phenotype
specific to the differentiation stages

In order to collect specific NK cells from each differentiation stages, Lin⁻ c-kit⁺ HSC (> 95%) cells, separated from mouse bone marrow, were cultured in the presence of SCF, Flt-3L and IL-7 for 6 days. Then, CD122⁺ pNK cells were separated and analyzed by flow cytometry. In the case of mNK cells (-OP9 or +OP9), IL-15 cells were cultured only or with OP9 stromal cells for 6 more days. The recovered cells were analyzed by flow cytometry (FIG. 1a). When the cells were cultured together with OP9 stromal cells, the number of mNK cell was increased (-OP9; 94% and +OP9; > 95%). Ly49 receptors on the surface of mNK cells play an important role in mNK cell functions and their expression is regulated by a signal transduction by the communication with other immune cells. In order to confirm whether or not the co-culture of HSC cells derived from bone marrow and stromal cells was essential for the expression of Ly49 receptors of mNK cells, mNK cells were cultured only or together with OP9 cells in the presence of IL-15 and then the expression of Ly49 was investigated (FIG. 1b). When the cells were cultured together with OP9 cells (+OP9), Ly49C/I and Ly49G2 were expressed in mNK cells. On the other hand, when the cells were cultured

independently (-OP9), neither Ly49C/I nor Ly49G2 were expressed. The results indicate that the co-culture of HSC cells and OP9 cells is essential for the maturation of NK cells. After investigating the expressions of CD122 and perforin genes according to the differentiation stages of NK cells, HSC cells were proved to become mature to NK cells during the differentiation (FIG. 1c).

<Example 3> SAGE(Serial analysis of gene expression)

Whole RNA was extracted from HSC cells prepared in the Example 2 and from NK differentiation stage specific cells (pNK and mNK). mRNA was separated and purified from 5 μ g of the whole RNA by using (dT)25 magnetic beads (Dynal A.S., Oslo, Norway). The mRNA, separated and purified by the oligo dT beads, was used as a template for the synthesis of cDNA by cDNA synthesis kit (Invitrogen, Life Technologies) using oligo (dT) primer that was 5'-biotinized and 3'-linked. According to the manufacturer's instructions (Invitrogen, Life Technologies), tag for SAGE was prepared from the cDNA by the method explained in the schematic diagram of FIG. 2. The cDNA was digested with restriction enzyme NlaIII and 3'-region was bound to magnetic beads (Dynal) coated with streptavidin. The tag was divided into two fractions, which were bound to

linkers (Invitrogen, Life Technologies) having NlaIII recognition site, respectively. Linker binding tag was digested with BsmFI. The isolated tag and the linker were treated with Pfu DNA polymerase to make blunt-end. The blunt-ends were linked together to form a ditag. PCR was performed to amplify the ditag by using biotin labeled SAGE primer (Invitrogen, Life Technologies). Then, the ditag was digested with NlaIII to be separate from linker. T4 DNA ligase was treated thereto to form a concatemer. The prepared concatemer was cloned into Sph I pre-digested pZero-1 vector (Invitrogen, Carlsbad, CA) (FIG. 2). Then, the cloning product was amplified by PCR using M13 forward primer represented by SEQ. ID. No. 1 and M13 backward primer represented by SEQ. ID. No. 2. Amplified positive colony was collected, and then the sequence was investigated by sequencing kit (Big-Dye sequencing kit) and nucleotide sequencer (ABI377 sequencer, Perkin-Elmer Applied Biosystems, Branchburg, NJ). The sequence of tag was identified by SAGE 300 soft ware.

<Example 4> SAGE data analysis

<4-1> Bioinformatical analysis

Reference SAGE-tag database was established from UniGene mouse database harboring most sequences expressed

in a mouse, which was filed in GenBank. SAGE tag was determined by (i) direction of each transcript, (ii) presence or absence of poly(A) signal (AATAAA or ATTAAA), (iii) presence or absence of poly A tail, and (iv) presence or absence of the last CATG cleavage site in a sequence. All SAGE tags extracted from reference sequences were used for the construction of reference SAGE database. Experimental SAGE tag was matched with reference SAGE database (<http://www.hpcl.cs.uchicago.edu/gist>). In order to identify a gene corresponding to each SAGE tag, a computer program SAGEmap (Lash A.E et al., 2000) was used.

<4-2> Analysis of clustering according to quantitative distribution of SAGE profile

A clustering computer program (cluster and treeview computer program, <http://rana.lbl.gov>) was used to investigate clustering of SAGE data obtained in the Example 4-1, based on other expressions and functional patterns shown during NK cell differentiation processes. Briefly, in each stage, different colors such as blue, green, yellow and red were marked according to the frequency (PERL script available upon request). Mid-point was included in the corresponding RGB value. According to

the colorful results, some tags showing clear and high expression were selected and let them apart from each other in panel. The remaining tags were re-arranged, placing lines showing similar expression patterns beside
5 in order to make gradual color change as a whole.

The increase or the decrease of gene expression during NK cell differentiation was investigated based on SAGE profiles of HSC, pNK, mNK(-OP) and mNK(+OP9) cells. As a result, as shown in FIG. 3a - FIG. 3f, the target
10 genes were clustered into 4 groups. Precisely, FIG. 3a presents a gene group whose expression was increased in HSC but decreased by the NK cell differentiation, FIG. 3b shows a gene group whose expression is high in pNK cells and FIG. 3c presents a gene group whose expression was
15 high in mNK(-OP9). FIG. 3d shows a gene group whose expression was gradually increased until it reached maximum in mNK(+OP9) cells. In particular, the gene group (FIG. 3b) showing the best expression in pNK cells includes many immune regulating genes such as lymphocyte
20 differentiation antibody, C-C chemokine receptor, tumor necrosis factor and interleukin-18 binding protein, etc, indicating that immune regulating factors play an important role in pNK cell differentiation. Next, based on the informed database, genes were classified by the
25 function of regulating the NK cell activity. FIG. 3e and

FIG. 3f show genes inhibiting and promoting the NK cell activity, respectively. In most cases, those genes are expressed in late stage of differentiation. Genes involved in the cell activation include many signal factors such as mitogen activated protein kinase, phospholipase A2, IL-2 receptor, chemokine receptor, etc.

<Example 5> Analysis of genes regulating each stage of NK cell differentiation

<5-1> Construction of SAGE library according to each stage of NK cell differentiation

Based on the results of SAGE in the Example 4, 4 different SAGE libraries were constructed according to each stage of NK cell differentiation (HSC, pNK, mNK(-OP9), mNK(+OP9)). From SAGE library of HSC, 19,830 unique transcripts were identified from total 44,998 tags, and among them, 12,899 specific genes were identified. From SAGE library of pNK, 17,745 unique transcripts were identified from total 40,771 tags, and among them, 11,684 specific genes were identified. Likewise, from SAGE library of mNK, 20,803 and 20,791 unique transcripts were each identified from 42,160 tags (mNK(-OP9)) and 42, 535 tags (mNK(+OP9)), and among them, 3,650 and 14,335

specific genes were identified respectively. On the whole, total 170,464 tags were identified from the above four SAGE libraries, from which 59,657 unique transcripts and 35,385 specific genes were identified. Among 59,657 unique transcripts, 77.9% were single copy, 16.8% showed 2-4 copies, 3.2% showed 5-9 copies, 1.9% had 10-99 copies, and just 0.2% had over 100 copies (Table 1).

【Table 1】

SAGE result according to each stage of NK cell differentiation

Cells according to each stage of differentiation	Number of tags	Number of unique transcripts	Number of specific genes
HSC	44,998	19,830	12,899
pNK	40,771	17,745	11,684
mNK(-OP9)	42,160	20,803	13,650
mNK(+OP9)	42,535	20,791	14,335
Total	170,464	59,657	35,385

The reflection of the expression patterns of genes known to have an effect on NK cell differentiation was also investigated based on the above result of SAGE. As a result, as expected, the numbers of mNK cell receptors such as granzyme (GenBank ID NM_013542), NKG2A (GenBank ID

AF106008), 2B4 (GenBank ID L19057), Ly49Q (GenBank ID AB033769) and CD94 (GenBank ID AF057714) were big in mNK cells but were not counted in HSC and pNK cells, either. IL-15 (GenBank ID U14332) was detected only in HSC and pNK cells. The expression of ID2 (GenBank ID BC006951) began from the stage of pNK cells (Table 2).

【Table 2】

SAGE result of differentiation related genes

Gene	HSC	pNK	mNK (-OP9)	mNK (+OP9)
Granzyme	0	0	508	664
NKG2A	0	0	6	3
NK receptor 2B4	1	0	17	17
NK receptor Ly-49Q	0	1	2	6
CD94	0	0	3	1
IL-15	3	3	0	0
Ly49G2	0	0	1	0
ID-2	0	7	5	9

<5-2> Analysis of differentiation stage specific genes expressed in each NK cell differentiation stage

It was reported that different genes were expressed according to NK cell differentiation stages, so that the

present inventors identified differentiation stage specific genes. For the statistical significance, genes at least 4-fold counted were grouped and presented in a table.

5

As a result, 15 genes were confirmed to be highly expressed in HSC (Table 3). In particular, interleukin-1 receptor associated kinase (IRAK) involved in the NK cell activation and signal transduction. In consideration of the report that the ability to induce cytotoxicity in NK cell caused by IL-18 and the generation of IFN- γ by activated NK cell were seriously damaged and decreased in IRAK-deficient mouse, the analysis of the present invention was correctly done.

15

【Table 3】

Gene	GenBank ID	HSC	pNK	MNK (-OP9)	MNK (+OP9)
Homeobox protein MIX	AF15457	28	0	0	0
Pre-pro-proteinase 3	U97073	28	0	0	0
Myeloblastosis (Myb) oncogene	M16499	11	1	0	1
Keratin complex 1, acidic, gene 13	NM_010662	9	0	0	0
PA-	AK002966	8	0	1	1

phosphatase related phosphoester ase					
Interleukin 1 receptor- associated kinase	AK009132	7	0	0	0
Gamma-parvin	BC011200	6	0	0	0
Forkhead- related transcriptio n factor 1C	AF330105	4	1	1	0
RIKEN cDNA 5730501N20 gene	AK017744	4	1	0	0
c-myc protein	X010223	4	0	0	1
Ribosomal protein L10A	AK002613	4	0	1	0
Oct 2b gene	X53654	4	0	0	0
Microlite	AK015601	4	0	0	0
Dihydrolipoa mide dihydrogenas e	BC003368	4	0	0	0
Tracle	U81030	4	0	0	0

And, 30 other genes were exceptionally expressed in pNK cell stage (Table 4). Among them, c-kit ligand was confirmed to be essential for the complete differentiation into mNK cells and so the progress from premature NK cells into mature NK cells was inhibited in the absence of c-kit signal transduction. It was also reported that 2-microglobulin is involved in the beginning of the

expression of Ly49 receptor and in the variety of NK cell receptors which are major regulators of NK cell differentiation. The expression of transformed Fc receptor affects the development and the function of NK cells, resulting in the decrease of the number of CD56+CD3- NK cells and further in cytopenia and other critical immunodeficiency syndroms. According to the result that genes known to regulate NK cell differentiation were expressed in the right stages as expected, the analysis of the present invention was correctly done.

【Table 4】

Gene	GenBank ID	HSC	pNK	MNK (-OP9)	MNK (+OP9)
Lysozyme	BC002069	14	1321	2	3
Ferritin H chain	BC012314	25	962	7	18
Brevican	X87096	7	259	1	1
Matrix metalloproteinase 12	BC019135	0	69	0	0
EIA-stimulated gene cellular inhibitor	AF084524	5	45	7	1
c-kit ligand	M64262	0	62	0	0
S100 calcium binding protein A9	BC027635	1	42	0	1
MPS1 protein	L20315	1	35	0	0

Transglutaminase 2	BC016492	0	25	1	1
Serum and glucocorticoid regulated protein kinase	AF139639	0	20	0	0
RIKEN cDNA 5830413L19	BC027496	0	18	0	0
Beta 2- microglobulin mRNA	M10416	0	17	0	0
Interferon- induced protein	BC003804	0	17	0	0
Milk fat globul membrane protein EGF factor 8	BC018577	3	16	0	1
Fc gamma receptor	M14215	3	15	1	1
Cell-surface glycoprotein p91	U83172	0	13	0	1
Arginase 1	BC050005	0	12	0	0
Tumor Necrosis factor receptor 1	M59378	1	12	0	2
Retinoid-induced serine carboxypeptidase	AF330052	2	11	0	0
Unidentified protein FLJ11000 homologue	BC023802	0	11	2	0
Interleukin-18 binding protein d precursor	AF110803	0	10	0	0
Chloride channel 7	AK009435	0	9	1	0
CD36 antigen	BC010262	0	8	0	0
Zink finger protein homologue	BC030186	1	8	1	0
Carbohydrate binding protein 35	J03723	0	7	3	0
C-type calcium	BC003218	0	7	0	0

dependent carbohydrate					
Lipoprotein lipase	NM_008509	0	7	0	0
v-maf lacertus fibrosarcoma oncogene	BC038256	0	6	0	0
Interleukin 7 receptor	NM_008372	0	5	0	0
Chemokine (C-C) receptor 1	BC011092	0	5	0	0
Neurophilin (MGD MGI:106206)	AK002673	0	5	0	0

In the meantime, 27 genes were identified from mNK cell stage (Table 5). Among them, Src family tyrosin kinase 'Fyn' is known to be involved in the activation of NK cell.

【Table 5】

Gene	GenBank ID	HSC	pNK	MNK (-OP9)	MNK (+OP9)
SERPINA3G	XM_127137	2	0	29	45
GABA-A receptor subunit 6	X51986	0	0	16	44
LAPTm5	U51239	5	4	18	25
G-protein signal regulator	BC049968	0	0	0	17
Decoy-stimulating factor GPI fixed mRNA	L41366	0	0	0	12
Y box protein 3	AK019465	0	0	10	17

Osteopontin precursor	J04806	0	1	2	14
Amyloid beta (A4) precursor protein-binding family	AK021331	2	0	5	12
T cell receptor beta subunit analogue	U63547	0	0	8	11
Immune related nucleotide 1	BC005577	0	0	9	0
Higher stage transcription factor 1	NM_009480	0	1	0	8
Olfactory receptor MOR267-7	NM_146714	0	0	0	8
Lymphocyte specific protein tyrosine kinase	M12056	0	0	7	1
Osteoclast cancer inhibitor	AB013898	1	1	0	7
Platelet active receptor homologue	BC024054	0	1	3	7
Natural killer cell protein 2-A1	AF016008	0	0	3	6
Unidentified protein MGC36662	BC023851	0	1	2	6
Semaphorin 6A precursor homologue	AK004390	0	0	6	2
Fyn proto-oncogene	BC032149	0	0	5	5
Neurofilament homologue, polypeptide	BC025872	0	0	2	5
Cornin	BC026634	1	1	6	2

homologue, actin binding protein 2A					
Solute transmitting family 6	BC015245	1	1	6	5
Temporary purine receptor P2Y10 homologue	AK020001	0	0	5	4
T cell receptor gamma chain	X03802	0	1	5	4
Poly A polymerase alpha	NM_011112	0	0	5	3
OPA-related protein OIP5 analogue	AK017825	0	0	5	1
Mytogen activated protein kinase 1 analogue	BC006708	1	0	5	4

<Example 6> Investigation of expression patterns of genes

by RT-PCR

Semiquantitative RT-PCR was performed to investigate
 5 expression patterns of other genes, based on SAGE data.
 Primers for the RT-PCR were prepared according to target
 genes. All PCR mixtures were heated at 95°C for 1 minute,
 and other PCR conditions were as follows; PCR with HSC and
 mNK cells was performed at 95°C for 1 minute, at 55°C for 1
 10 minute and at 72°C for 2 minutes, and PCR with premature
 NK cells was performed at 95°C for 1 minute, at 60°C for 1

minute and at 72°C for 2 minutes, which were repeated 28 or 32 cycles, and then extension followed at 72°C for 10 minutes. The amplified PCR products were electrophorezed and stained with ethidium bromide.

- 5 Gamma-parvin: SEQ. ID. No 3 and No 4,
 Forkhead-related transcription factor 1c (Foxp1c):
SEQ. ID. No 5 and No 6,
 c-myc protein: SEQ. ID. No 7 and No 8,
 Keratin complex (KC) 1: SEQ. ID. No 9 and No 10,
10 PA-phosphatase related phosphoesterase (PA-PRP): SEQ.
ID. No 11 and No 12,
 Interleukin 1 receptor-associated kinase (IRAK): SEQ.
ID. No 13 and No 14,
 Ribosomal protein L10A: SEQ. ID. No 15 and No 16,
15 Pre-pro-proteinase 3: SEQ. ID. No 17 and No 18,
 Myeloblastosis oncogene: SEQ. ID. No 19 and No 20,
 Carbohydrate binding protein (CBP) 35: SEQ. ID. No
21 and No 22,
 IL-7 receptor: SEQ. ID. No 23 and No 24,
20 Lipoprotein lipase (LPL): SEQ. ID. No 25 and SEQ. ID.
No 26,
 Ferritin H chain: SEQ. ID. No 27 and No 28,
 Matrix metalloproteinase (MMP) 12: SEQ. ID. No 29
and No 30,
25 Regulator of G-protein signaling (RGS): SEQ. ID. No

31 and No 32,

Serpina 3G: SEQ. ID. No 33 and No 34,

Purinergic receptor P2Y: SEQ. ID. No 35 and No 36,

Lymphocyte-specific protein tyrosin kinase (PTK):

5 SEQ. ID. No 37 and No 38,

Semaphorin 6A precursor: SEQ. ID. No 39 and No 40,

CD122: SEQ. ID. No 41 and No 42,

Perforin: SEQ. ID. No 43 and No 44,

Beta-actin: SEQ. ID. No 45 and No 46

10

As a result, 9 genes, for example gamma-parvin, forkhead-related transcription factor 1c (Foxp1c), c-myc, pre-pro-proteinase 3, etc, were specifically expressed in HSC (FIG. 4a). IL-7R and matrix metalloproteinase 12
15 (MMP12) were exceptionally expressed in pNK cells (FIG. 4b). Purinergic receptor P2Y10 and lymphocyte-specific protein tyrosin kinase (PTK) were unusually expressed in mNK cells (FIG. 4c).

20 <Example 7> Effect of LPL on NK cell differentiation stages

In the above Example 4, it was confirmed that lipoprotein lipase (referred as 'LPL' hereinafter) represented by SEQ. ID. No 47 was over-expressed in pNK

cells during NK cell differentiation among many differentiation stage specific genes. LPL promotes NK cell proliferation but inhibits spontaneous cytotoxicity and activity of lymphokine-activated killer (LAK). In order to confirm whether pNK-specific expression of LPL was required for the differentiation into mNK cells, HSC cells were cultured for 6 days, which were then treated with IL-15 and LPL in the absence of OP9 stromal cells, followed by measuring the percentage of NK cells.

As a result, the NK cell percentage was increased more when HSC was treated with IL-15 and LPL together than when it was treated with IL-15 only (NK1.1+ NKG2A/C/E+ cell; 50% when it was treated with IL-15 only versus 71% and 86% each when treated with IL-15 and 250 ng/ml of LPL together and when treated with IL-15 and 500 ng/ml of LPL together) (FIG. 4d). The above results indicate that LPL plays an important role in the differentiation from pNK cells into mNK cells and the search of genes regulating NK cell differentiation was correctly done in the present invention.

【Industrial Applicability】

As explained hereinbefore, the method of the present invention for searching genes involved in the regulation of differentiation from stem cells into natural killer cells, in addition to SAGE, is very useful for identifying a novel gene having unfamiliar functions.

【Sequence List Text】

Nucleotide sequences represented by SEQ. ID. No 1 and No 2 are the primer sequences used for the PCR in the Example 3.

Nucleotide sequences represented by SEQ. ID. No 3 and No 4 are the primer sequences used for the RT-PCR with gamma-parvin in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 5 and No 6 are the primer sequences used for the RT-PCR with forkhead-related transcription factor 1c in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 7 and No 8 are the primer sequences used for the RT-PCR with c-myc protein in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 9 and No 10 are the primer sequences used for the RT-PCR with keratin complex (KC) 1 in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 11 and No 12 are the primer sequences used for the RT-PCR with PA-phosphatase related phosphoesterase (PA-PRP) in

the Example 6.

Nucleotide sequences represented by SEQ. ID. No 13 and No 14 are the primer sequences used for the RT-PCR with interleukin 1 receptor-associated kinase (IRAK) in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 15 and No 16 are the primer sequences used for the RT-PCR with ribosomal protein L10A in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 17 and No 18 are the primer sequences used for the RT-PCR with pre-pro-proteinase 3 in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 19 and No 20 are the primer sequences used for the RT-PCR with myeloblastosis oncogene in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 21 and No 22 are the primer sequences used for the RT-PCR with carbohydrate binding protein (CBP) 35 in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 23 and No 24 are the primer sequences used for the RT-PCR with IL-7 receptor in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 25 and No 26 are the primer sequences used for the RT-PCR with lipoprotein lipase (LPL) in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 27

and No 28 are the primer sequences used for the RT-PCR with ferritin H chain in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 29 and No 30 are the primer sequences used for the RT-PCR
5 with matrix metalloproteinase (MMP) 12 in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 31 and No 32 are the primer sequences used for the RT-PCR with Regulator of G-protein signaling (RGS) in the Example 6.

10 Nucleotide sequences represented by SEQ. ID. No 33 and No 34 are the primer sequences used for the RT-PCR with serpina 3G in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 35 and No 36 are the primer sequences used for the RT-PCR
15 with purinergic receptor P2Y in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 37 and No 38 are the primer sequences used for the RT-PCR with Lymphocyte-specific protein tyrosin kinase (PTK) in the Example 6.

20 Nucleotide sequences represented by SEQ. ID. No 39 and No 40 are the primer sequences used for the RT-PCR with semaphorin 6A precursor in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 41 and No 42 are the primer sequences used for the RT-PCR
25 with CD122 in the Example 6.

Nucleotide sequences represented by SEQ. ID. No 43 and No 44 are the primer sequences used for the RT-PCR with Perforin in the Example 6.

5 Nucleotide sequences represented by SEQ. ID. No 45 and No 46 are the primer sequences used for the RT-PCR with beta-actin in the Example 6.

Nucleotide sequence represented by SEQ. ID. No 47 is the nucleotide sequence of lipoprotein lipase.

10 Nucleotide sequence represented by SEQ. ID. No 48 is the amino acid sequence of a mouse protein.

15 Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

20

【CLAIMS】**【Claim 1】**

A differentiation regulating agent, which regulates differentiation from stem cells into natural killer cells, containing one or more genes, as an effective ingredient, selected from a group consisting of homeobox protein MIX (AF15457), pre-pro-proteinase 3 (U97073), myeloblastosis (Myb) oncogene (M16499), keratin complex 1, acidic, gene 13 (NM_010662), PA-phosphatase related phosphoesterase (AK002966), gamma-parvin (BC011200), forkhead-related transcription factor 1C (AF330105), RIKEN cDNA 5730501N20 gene (AK017744), c-myc protein (X010223), ribosomal protein L10A (AK002613), Oct 2b gene (X53654), microlite (AK015601), dihydrolipoamide dihydrogenase (BC003368), tracle (U81030), lysozyme (BC002069), ferritin H chain (BC012314), brevican (X87096), matrix metalloproteinase 12 (BC019135), EIA-stimulated gene cellular inhibitor (AF084524), S100 calcium binding protein A9 (BC027635), MPS1 protein (L20315), transglutaminase 2 (BC016492), serum and glucocorticoid regulated protein kinase (AF139639), RIKEN cDNA 5830413L19 (BC027496), interferon-induced protein (BC003804), milk fat globul membrane protein EGF factor 8 (BC018577), cell-surface glycoprotein p91 (U83172), arginase 1 (BC050005), tumor

necrosis factor receptor 1 (M59378), retinoid-induced
serine carboxypeptidase (AF330052), FLJ11000 homologue
(BC023802), interleukin-18 binding protein d precursor
(AF110803), chloride channel 7 (AK009435), CD36 antigen
5 (BC010262), zink finger protein homologue (BC030186),
carbohydrate binding protein 35 (J03723), C-type calcium
dependent carbohydrate (BC003218), lipoprotein lipase
(NM_008509), v-maf lacertus fibrosarcoma oncogene
(BC038256), interleukin 7 receptor (NM_008372), chemokine
10 (C-C) receptor 1 (BC011092), neurophilline
(MGD|MGI:106206) (AK002673), SERPINA3G (XM_127137), GABA-A
receptor subunit 6 (X51986), LAPtm5 (U51239), G-protein
signal regulator (BC049968), decoy-stimulating factor GPI
fixed mRNA (L41366), Y box protein 3 (AK019465),
15 osteopontin precursor (J04806), amyloid beta (A4)
precursor protein-binding family (AK021331), T cell
receptor beta subunit analogue (U63547), immune related
nucleotide 1 (BC005577), higher stage transcription factor
1 (NM_009480), olfactory receptor MOR267-7 (NM_146714),
20 lymphocyte specific protein tyrosine kinase (M12056),
osteoclast cancer inhibitor (AB013898), platelet active
receptor homologue (BC024054), natural killer cell protein
2-A1 (AF016008), unidentified protein MGC36662 (BC023851),
semaphorin 6A precursor homologue (AK004390),
25 neurofilament homologue polypeptide (BC025872), cornin

homologue actin binding protein 2A (BC026634), solute
transmitting family 6 (BC015245), temporary purine
receptor P2Y10 homologue (AK020001), T cell receptor gamma
chain (X03802), poly A polymerase alpha (NM_011112), OPA-
5 related protein OIP5 analogue (AK017825) and mytogen
activated protein kinase 1 analogue (BC006708).

【Claim 2】

A differentiation regulating agent, which regulates
10 differentiation from stem cells into premature natural
killer cells, containing one or more genes, as an
effective ingredient, selected from a group consisting of
homeobox protein MIX (AF15457), pre-pro-proteinase 3
(U97073), myeloblastosis (Myb) oncogene (M16499), keratin
15 complex 1, acidic, gene 13 (NM_010662), PA-phosphatase
related phosphoesterase (AK002966), gamma-parvin
(BC011200), forkhead-related transcription factor 1C
(AF330105), RIKEN cDNA 5730501N20 gene (AK017744), c-myc
protein (X010223), ribosomal protein L10A (AK002613), Oct
20 2b gene (X53654), microlite (AK015601), dihydrolipoamide
dihydrogenase (BC003368) and tracle (U81030).

【Claim 3】

A differentiation regulating agent, which regulates
25 differentiation from premature natural killer cells into

mature natural killer cells containing one or more genes,
as an effective ingredient, selected from a group
consisting of lysozyme (BC002069), ferritin H chain
(BC012314), brevican (X87096), matrix metalloproteinase 12
5 (BC019135), EIA-stimulated gene cellular inhibitor
(AF084524), S100 calcium binding protein A9 (BC027635),
MPS1 protein (L20315), transglutaminase 2 (BC016492),
serum and glucocorticoid regulated protein kinase
(AF139639), RIKEN cDNA 5830413L19 (BC027496), interferon-
10 induced protein (BC003804), milk fat globul membrane
protein EGF factor 8 (BC018577), cell-surface
glycoprotein p91 (U83172), arginase 1 (BC050005), tumor
necrosis factor receptor 1 (M59378), retinoid-induced
serine carboxypeptidase (AF330052), FLJ11000 homologue
15 (BC023802), interleukin-18 binding protein d precursor
(AF110803), chloride channel 7 (AK009435), CD36 antigen
(BC010262), zink finger protein homologue (BC030186),
carbohydrate binding protein 35 (J03723), C-type calcium
dependent carbohydrate (BC003218), lipoprotein lipase
20 (NM_008509), v-maf lacertus fibrosarcoma oncogene
(BC038256), interleukin 7 receptor (NM_008372), chemokine
(C-C) receptor 1 (BC011092) and neurophilline
(MGD|MGI:106206).

25 **【Claim 4】**

A differentiation regulating agent, which regulates differentiation of mature natural killer cells, containing one or more genes, as an effective ingredient, selected from a group consisting of SERPINA3G (XM_127137), GABA-A receptor subunit 6 (X51986), LAPTM5 (U51239), G-protein signal regulator (BC049968), decoy-stimulating factor GPI fixed mRNA (L41366), Y box protein 3 (AK019465), osteopontin precursor (J04806), amyloid beta (A4) precursor protein-binding family (AK021331), T cell receptor beta subunit analogue (U63547), immune related nucleotide 1 (BC005577), higher stage transcription factor 1 (NM_009480), olfactory receptor MOR267-7 (NM_146714), lymphocyte specific protein tyrosine kinase (M12056), osteoclast cancer inhibitor (AB013898), platelet active receptor homologue (BC024054), natural killer cell protein 2-A1 (AF016008), unidentified protein MGC36662 (BC023851), semaphorin 6A precursor homologue (AK004390), neurofilament homologue polypeptide (BC025872), cornin homologue actin binding protein 2A (BC026634), solute transmitting family 6 (BC015245), temporary purine receptor P2Y10 homologue (AK020001), T cell receptor gamma chain (X03802), poly A polymerase alpha (NM_011112), OPA-related protein OIP5 analogue (AK017825) and mytogen activated protein kinase 1 analogue (BC006708).

【Claim 5】

The differentiation-regulating agent as set forth in anyone of claim 1 to claim 4, wherein the differentiation-regulating agent is used for the treatment of cancer.

5

【Claim 6】

The differentiation regulating agent as set forth in claim 5, wherein the cancer is selected from a group consisting of breast cancer, melanoma and lung cancer.

10

【Claim 7】

A screening method for a gene regulating the differentiation from stem cells into natural killer cells comprising the following steps:

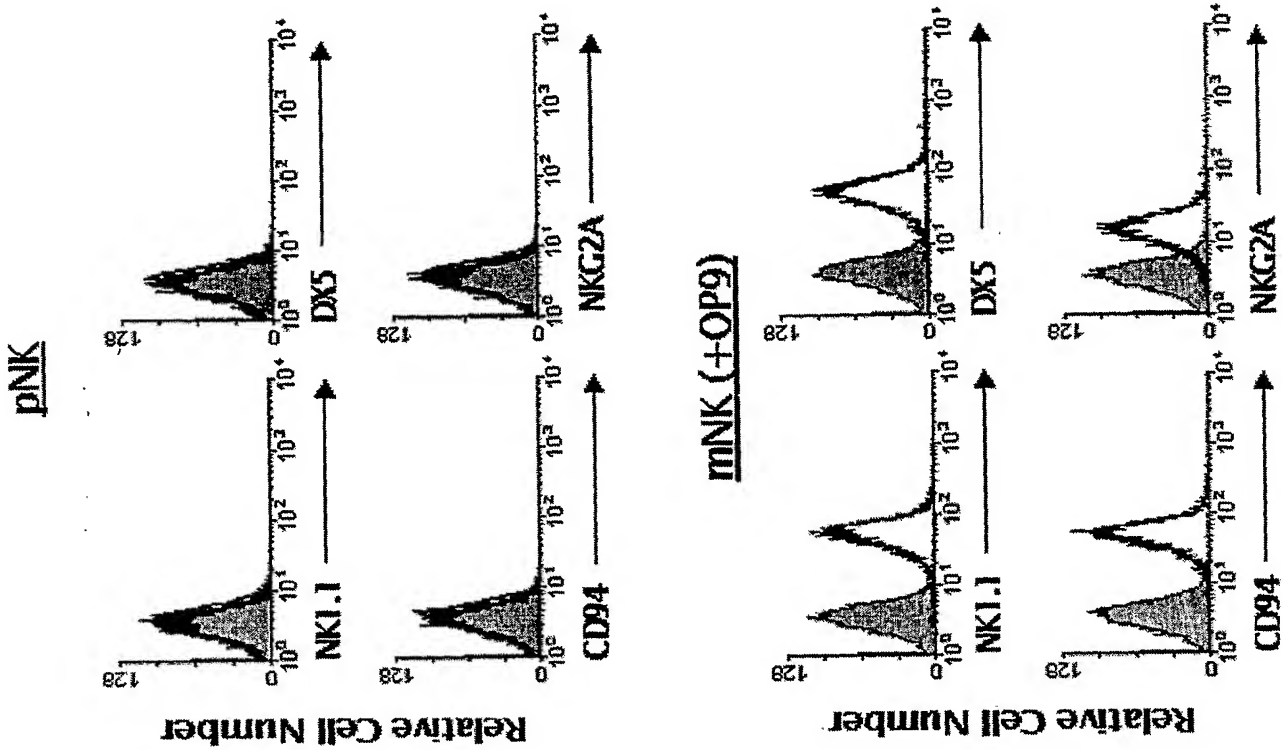
15 1) Synthesizing cDNA after separating whole RNA from cells;

 2) Separating tag after digesting the cDNA of the step 1;

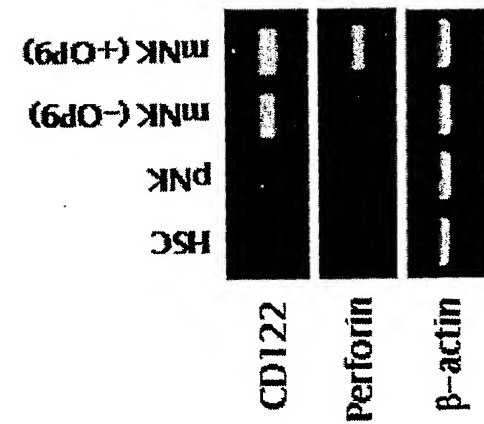
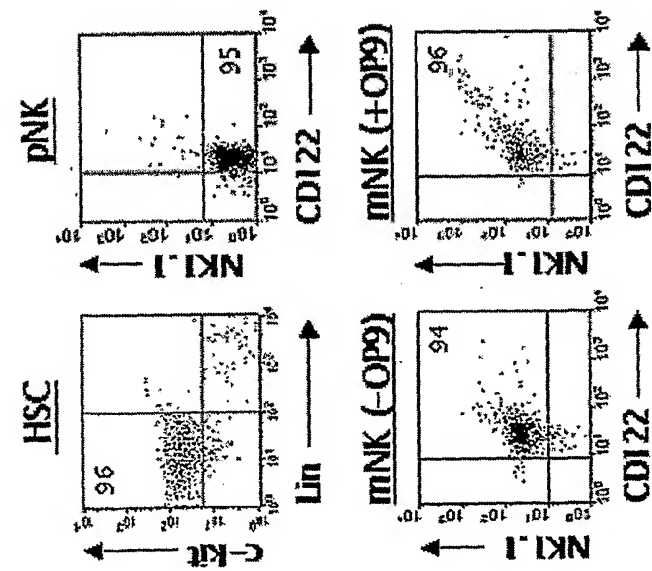
 3) Connecting each tag separated in the step 2 and then analyzing nucleotide sequence thereof; and

20 4) Quantifying the expression of the gene, based on the analyzed nucleotide sequence above, by using SAGE (Serial Analysis of Gene expression) analyzing program.

1/6
H H
H 1



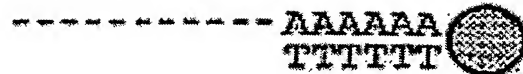
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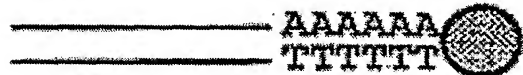
SUBSTITUTE SHEET

2/6
도 2

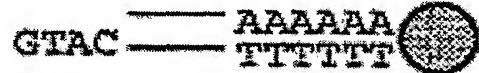
1. 올리고 dT 자석 비드로
RNA 샘플을 결합



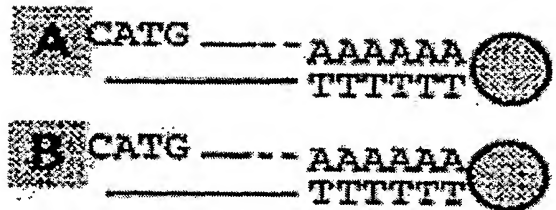
2. 이중 가닥 cDNA의 합성



3. NlaIII로 절단하여 하나의
태그 말단 형성



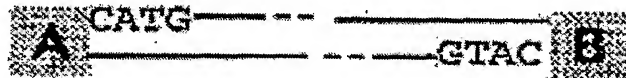
4. NlaIII 제한효소 인식부위를
포함하는 어댑터 A, 어댑터 B로
샘플의 절반씩을 연결



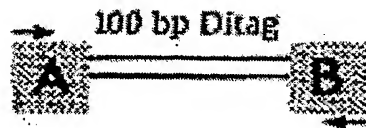
5. BsmFI 으로 절단하여 ~50bp 태그 형성
(40bp 어댑터/14bp 태그)



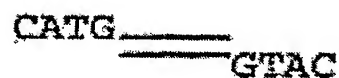
6. 5' 오버행을 채워서
~100bp의 태그형성



7. 이중태그 프라이머 1 및 2를 사용하여
PCR 증폭



8. NlaIII로 40bp 어댑터를 절단하여
26bp 이중태그 생산



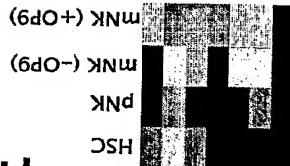
4/6
H 3

HSC	PNK	MNK (-OP9)	MNK (+OP9)	Unigene Genebank	ID	Gene Name	Unigene Genebank	ID	Gene Name
				Mm.13020	NM_009438	ribosomal protein L13a	AK014294	AK014294	unnamed protein product; RIBOSOMAL PROTEIN 5271
				Mm.15085	BC002055	Unknown protein for MGC:5156	BC010367	BC010367	similar to RNA polymerase II transcriptional
				Mm.20149	AK013984	unknown EST (CBJAW553045)	BC010791	BC010791	suppressor of initiator codon mutations
				Mm.213045	BC023508	Similar to heterogeneous nuclear	AF457251	AF457251	cyclin arla-5a
				Mm.221288	BC028949	Similar to RIKEN cDNA 9030524 C23 gene	Mm.321	AF515708	osteopontin
				Mm.1034	BC005605	protein phosphatase 2 (formerly 2A, regulatory	Mm.28349	BC013640	Similar to NIPPE5 protein
				Mm.889	BC003820	Similar to RAS-related C3 botulinum substrate 1	Mm.195616	AK019465	Y-box protein 3 (MGD MGC:1095351)
				Mm.30854	BC004747	Similar to Knappel associated box (KRAB) zinc	Mm.14255	AK021331	amylid beta (A4) precursor protein-binding, family
				Mm.196581	BC005709	Similar to mitogen activated protein kinase 1	Mm.216195	AF485451	from a Homo sapiens melanoma cell line infected
				Mm.22435	BC003551	Similar to RIKEN cDNA 5730410D03 gene	Mm.20183	AK021021	unnamed protein product; hypothetical protein
				Mm.20051B	BC015245	solute carrier family 6 (neurotransmitter)	Mm.205030	BC023898	Similar to eukaryotic translation initiation
				Mm.22506	BC027314	G7e protein	Mm.3785	AK012612	unknown EST (CBJAW50327)
				Mm.20491	AB041653	hypothetical protein	Mm.142195	AK006570	unnamed protein product; putative
				Mm.35837	AK005111	weakly similar to PPI065 [Homo sapiens]	Mm.204990	BC011285	Unknown protein for MGC:7004
				Mm.22570	AK004719	unnamed protein product; putative	Mm.27880	BC026856	CUG triplet repeat RNA binding protein 2
				Mm.171547	BC026594	Similar to coronin, actin binding protein, 2A	Mm.195555	BC023905	Similar to SKB1 homolog (S. pombe)
				Mm.148782	BC010215	pleckstrin homology domain-containing, family A	Mm.3380	AK014590	unnamed protein product; KINISIN HEAVY CHAIN
				Mm.95677	AK017659	unnamed protein product; hypothetical Serine-rich	Mm.123648	BC024054	Similar to platelet activating receptor homolog
				Mm.14145	X03802	T-cell receptor gamma chain	Mm.3420	BC021717	no description
				Mm.22061	BC025800	sema domain, immunoglobulin domain (Ig)	Mm.4143	D78188	scid lung fibroblast cell line established by SV40
				Mm.2389	L17059	All-1 protein	Mm.143830	BC013516	alpha actinin 4
				Mm.15479	NM_013543	estradiol 17 beta-dehydrogenase B	Mm.205421	BC021400	Unknown protein for IMAGE:5101040
				Mm.27094	AK009218	unnamed protein product; NUCLEAR PROTEIN SKIP	Mm.24138	BC005576	Similar to Cell surface glycoprotein CD44
				Mm.59069	AF155546	hypothetical protein	Mm.10702	AK013924	calcyclin binding protein (MGD MGC:1270835)
				Mm.21606	AB045114	reticulon 3	Mm.30177	AK004388	unnamed protein product; hypothetical protein
				Mm.12193	BC003885	Similar to GDS ribosomal protein L30 isolog	Mm.103551	AK011125	unnamed protein product; hypothetical Tip-Asp (WD)
							Mm.27818	BC002233	unnamed protein product; putative
							Mm.4848	BC032149	Unknown protein for IMAGE:3491494
							Mm.88061	AK013831	Fyn proto-oncogene
							Mm.154503	AK014474	unnamed protein product; putative
							Mm.27194	AK009430	unnamed protein product; hypothetical Vacuolar
							Mm.22358	AK008537	DNA segment, Chr 7, Roswell Park 2 complex
							Mm.43760	BC010484	DNA-DIRECTED RNA POLYMERASE III 12.5 KOA
							Mm.200372	BC019444	signal peptidase complex (18kD)
									coronin, actin binding protein 1C

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E 3

E



Unigene ID	Genebank ID
BC002055	benzodiazepine receptor
Z36233	sialoadhesin
BC029900	serine protease inhibitor 6
AF143539	decay accelerating factor
AF106008	NK cell protein group 2-A1
AB033769	NK receptor Ly-49Q
AF110803	IL-18 binding protein d precursor

Gene Name

inhibition of NK activity
functions as an inhibitory receptor in human NK cells
suppression of exocytosis(perforin and granzyme) from NK cells
inhibition of human NK cytotoxicity
an inhibitory receptors
predicted to function as an inhibitory receptor
inhibition of NK cells responses

F



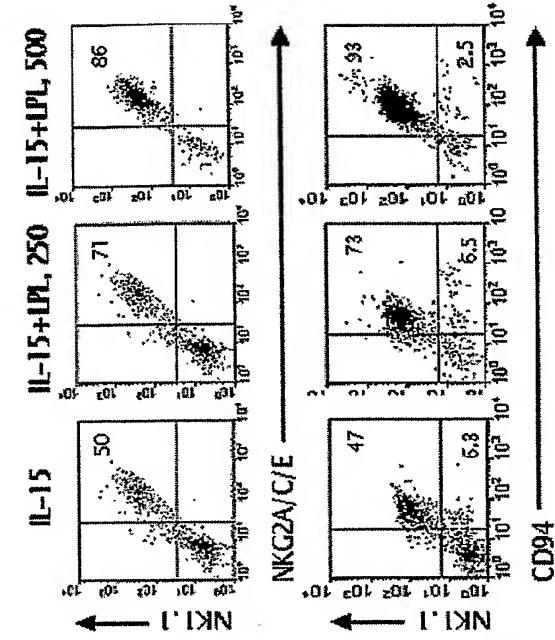
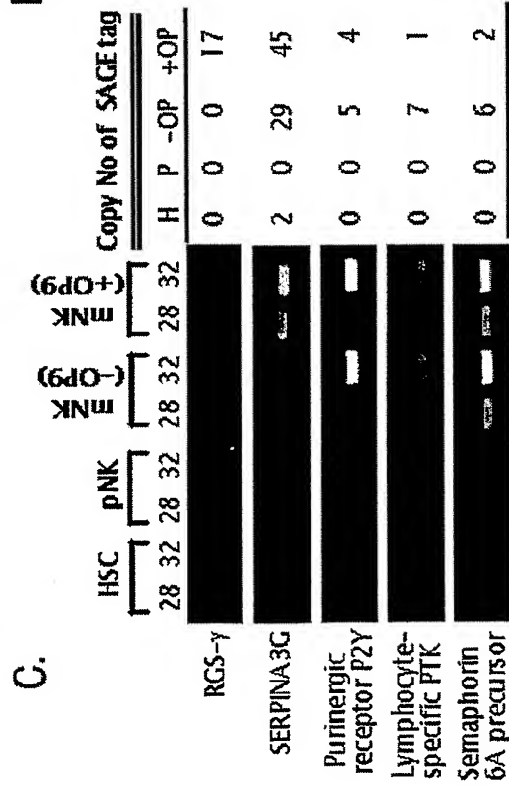
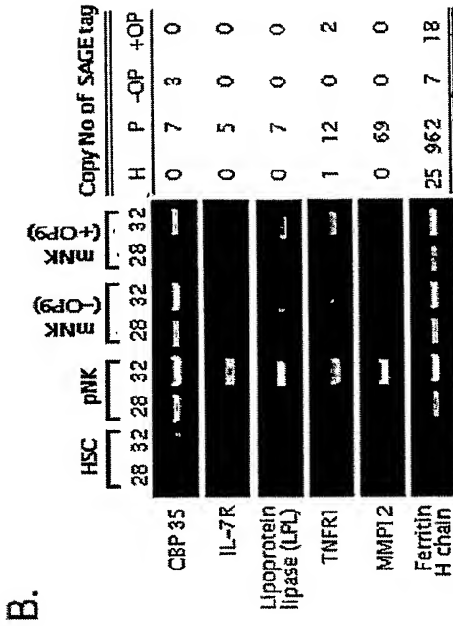
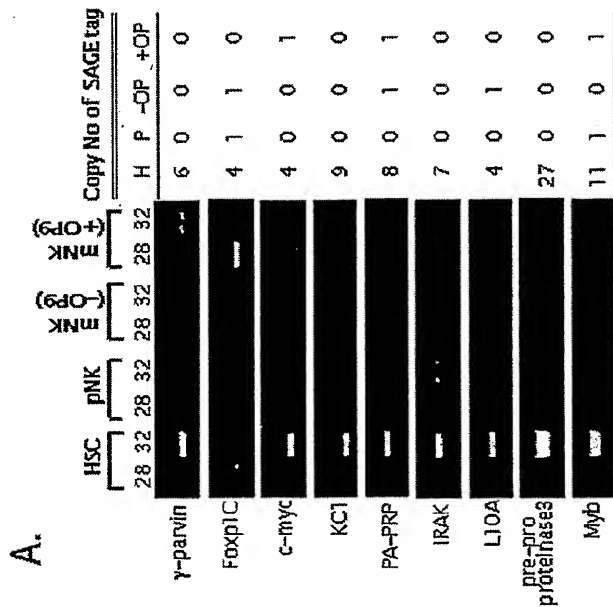
Unigene ID	Genebank ID
X01028	c-myc protein
BC005676	CD44
AF013274	type I interferon receptor
L24495	CD27
BC006708	mitogen activated protein kinase 1
AF082803	NK cell receptor 2B6 splice variant
U12889	L49SH
BC039347	phospholipase A2, group IVB
AJ440756	IL-1 receptor-associated kinase 2
M60778	lymphocyte function-associated molecule-1- α
NM1007589	calmodulin 2
AF054581	IL-2 receptor
AF515708	osteopontin
BC025597	Ca ²⁺ /calmodulin-dependent protein kinase II γ
BC031121	protein kinase C, ϵ
BC004714	heat shock protein, 70 kDa 2
L29480	serine/threonine kinase
M27255	p53fyn
BC029727	zeta-chain (TCR) associated protein kinase
X53584	mature H5T50 protein
AB052122	protein kinase C θ 2
BC030928	mitogen activated protein kinase kinase 11
AB032200	granzyme K
BC012558	similar to chemokine (C-X3-C) receptor 1
AF030313	NK2D
L274683	dipeptidyl peptidase precursor
X94151	MIP-1 alpha receptor
M14222	cathepsin B proteinase
BC004009	signal transducer and activator of transcription 1
AF213389	ATP-binding cassette protein
BC011092	chemokine (C-C) receptor 1
M10416	beta-2 microglobulin mRNA

Gene Name

activates NK cytotoxicity
upregulates NK cytotoxic activity
increases NK cytotoxicity
mediates activation of murine NK cells
involvement in NK lysis of tumor cells
activates NK cell mediated cytotoxicity and induces secretion of IFN- γ
activating natural killer receptor
involvement in the cytotoxic functions of rat NK cells
induction of NK cytotoxicity by IL-18
required for IL-12-induced NK cell cytotoxicity
induction of NK cells activation
NF-kappa B activation in perforin expression of NK cells
upregulated in activated murine NK cells
induces release of perforin and granzyme and secretion of IFN- γ
stimulates NK activity
interacts with NK receptor CD94 and stimulates NK activity
activates NK cytotoxicity
required NK cell-mediated lysis of target cells
Engagement of the NK cell IgC1c receptor results in tyrosine phosphorylation of the ζ chain.
interferes with CD94/NKG2A inhibitory receptor recognition
stimulates NK activity
activates NK cell function
triggers DNA fragmentation
Dual functions of fractalkine/CX3C ligand 1 in trafficking of perforin/granzyme Bt cytotoxic effector lymphocytes
triggers cytotoxicity in mouse NK cells
requirement for the intracellular processing of granzyme A and granzyme B
increases NK cytotoxicity
protect NK cells from self-destruction after degranulation.
requirements for NK cell functions
recruitment and activation of NK cells
recruitment of NK cells
enhancement of NK cell-mediated lysis via BACE1/NK2D interactions

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 4



SUBSTITUTE SHEET

SEQUENCE LISTING

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	Asp Phe Ser Asp Ile Glu Ser Lys Phe Ala Leu Arg Thr Pro Glu Asp				
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	aca gct gag gac act tgt cat ctc att cct gga tta gca gac tot gtg				192
	Thr Ala Glu Asp Thr Cys His Leu Ile Pro Gly Leu Ala Asp Ser Val				
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	Ala Ala Leu Tyr Lys Arg Glu Pro Asp Ser Asn Val Ile Val Val Asp				
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Ser Asn Cys His Phe Asn His Ser Ser Lys Thr Phe Val Val Ile His

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Gly Trp Thr Val Thr Gly Met Tyr Glu Ser Trp Val Pro Lys Leu Val

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Trp Leu Tyr Arg Ala Gln Gln His Tyr Pro Val Ser Ala Gly Tyr Thr

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Glu Phe Lys Tyr Pro Leu Asp Asn Val His Leu Leu Gly Tyr Ser Leu

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Pro Gly Cys Asn Ile Gly Glu Ala Ile Arg Val Ile Ala Glu Arg Gly

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470

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/000188

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C12N 15/12**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/12, A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI PubMed, Esp@cenet, CA "differentiation regulating agent, stem cell, natural killer cell"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUO W. et al., 'A human Mix-like homeobox gene MIXL shows functional similarity to Xenopus Mix.1', In: Blood, 2002, Vol. 100(1), pp. 89-95 See the whole document	1-7
A	DAVIDSON A.J. & ZON L.I., 'Turning mesoderm into blood: the formation of hematopoietic stem cells during embryogenesis', In: Curr. Top. Dev. Biol., 2000, Vol. 50, pp. 45-60 see the whole document	1-7
A	OGAWA M. et al., 'Expression of a 4-integrin defines the earliest precursor of hematopoietic cell lineage diverged from endothelial cells', In: Blood, 1999, Vol. 93(4), pp. 1168-1177 see the whole document	1-7
A	ALLEN R.D. et al., 'c-Myb is essential for early T cell development', In: Genes & Dev., 1999, Vol. 13(9), pp. 1073-1078 see the whole document	1-7
A	KLEFSTROM J. et al., 'c-Myc and E1A induced cellular sensitivity to activated NK cells involves cytotoxic granules as death effectors', In: Oncogene, 1999, Vol. 18(13), pp. 2181-2188 see the whole document	1-7

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 MAY 2005 (20.05.2005)

Date of mailing of the international search report

20 MAY 2005 (20.05.2005)

Name and mailing address of the ISA/KR



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Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHO, YOUNG GYUN

Telephone No. 82-42-481-8132



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2005/000188

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



in written format



in computer readable form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in computer readable form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: